

Specification

Title of the invention

A substance for accelerating nucleotide chain exchange  
5 reaction

Field of the invention

[0001]

The invention relates to a cationic polymer preparation  
10 (composition) for use in manipulation of a double stranded DNA  
or RNA. Particularly, the invention relates to a cationic  
polymer preparation used to exchange a nucleotide sequence at  
a specific site of a double stranded DNA or RNA for its  
homologous nucleotide sequence (a sequence that is  
15 complementary to a sequence of the other DNA or RNA strand).

Background of the invention

[0002]

A various types of nucleic acid-acting proteins are involved  
20 in the control of structural transition of nucleic acid hybrids  
within an organism.

[0003]

The nucleocapsid (NC) protein in retrovirus transfers DNA  
chain from one hybrid to more stable hybrid and plays some roles  
25 in viral infection ((a) Z. Tsuchihashi and P. O. Brown, J. Virol.

68, 5863(1994); (b) M. Lapadat-Tapolsky, C. Pornelle, C. Borie and J-L. Darlix, Nucleic Acids Res. 23, 2434 (1995)). NC protein has an activity to stimulate destruction and reassembly of base pairs, and acts as a nucleic acid chaperon to help appropriate hybridization of nucleic acids. ((a) W. Wu, L. E. Henderson, T. D. Copeland, R. J. Gorelick, W. J. Bosche, A. Rein and J. G. Levin, J. Virol. 70, 7132 (1996); (b) X. Ji, G. J. Klarmann and B. D. Preston, Biochemistry 35, 132 (1996); (c) V. Tanchou, C. Gabus, V. Rogemond and J-L. Darlix, J. Mol. Biol. 252, 563(1995)).

[0004]

RecA protein has a multivalent binding site to DNA and forms an intermediate complex with double stranded DNA (dsDNA) and single stranded DNA (ssDNA). ((a) J. W. Roberts, C. W. Roberts, N. L. Craig and E. M. Phizicky, Cold Spring Harbor Symp. Quant. Biol. 43, 917 (1979); (b) T. Shibata, C. DasGupta, R. P. Cunningham and C. M. Radding, Proc. Natl. Acad. Sci. USA 76, 1638 (1979); (c) K. McEntee, G. M. Weinstock and I. R. Lehman, Proc. Natl. Acad. Sci. USA 76, 2615 (1979); (d) E. Cassuto, S. C. West, J. Mursalim, S. Conlon and P. Howard-Flanders, Proc. Natl. Acad. Sci. USA 77 and 3962 (1980)).

[0005]

Since the sequence specificity and assembly property of a nucleic acid molecule are fully elucidated, they can be used for a molecular construction having a desirable structure at

a molecular level. A various types of molecules such as a DNA molecule machine ((a) B. Yurke, A. J. Turberfield, A. P. Mills Jr., F. C. Simmel and J. L. Neumann, *Nature* 406, 605 (2000); (b) H. Yan, X. Zhang, Z. Shen and N. C. Seeman, *Nature* 415 and 62 (2002)), a nano assembly ((a) B. Liu, N. B. Leontis and N. C. Seeman, *Nanobiology* 3, 177 (1994); (b) E. Winfree, F. Liu, L. A. Wenzler and N. C. Seeman, *Nature* 394, 539 (1998)), and a molecule computer ((a) L. M. Adleman, *Science* 266, 1021 (1994); (b) K. Sakamoto, H. Gouzu, K. Komiya, D. Kiga, S. Yokoyama, T. Yokomori, and M. Hagiya, *Science* 288, 1223 (2000)) are proposed. Therefore, an artificial substance that can manipulate structural transition of a nucleic acid molecule will be a useful tool not only in the biotechnology field but in the nanotechnology field.

[0006]

The inventors already have reported interaction between cationic comb-type copolymer (Cationic comb-type copolymers: CCCs) comprising cationic poly (L-lysine) (PLL) backbone and water-soluble side chain of dextran and DNA (A. Maruyama, M. Katoh, T. Ishihara and T. Akaike, *Bioconjugate Chem.* 8, 3 (1997); A. Maruyama, H. Watanabe, A. Ferdous, M. Katoh, T. Ishihara and T. Akaike, *Bioconjugate Chem.* 9, 292 (1998)). A comb-type copolymer with a higher degree of grafting accelerates DNA hybridization (H. Torigoe, A. Ferdous, H. Watanabe, T. Akaike and A. Maruyama, *J. Biol. Chem.* 274, 6161

(1999); A. Ferdous, T. Akaike and A. Maruyama, Bioconjugate Chem. 11, 520 (2000)), and stabilizes the double strand and ternary strand of DNA. Furthermore, it was proved that CCCs stimulate the DNA strand exchange reaction between dsDNA and ssDNA homologous thereto, and accelerates the rate more than spermine and N,N,N-trimethyl hexadecyl ammonium bromide (cetyl trimethyl ammonium bromide: CTAB) (50,000 fold at 37°C) (W. J. Kim, T. Ishihara, T. Akaike and A. Maruyama, Chem. Eur. J. 7, 176 (2001)). The copolymer is shown to have nucleic acid chaperon activity and useful in nucleic acid analysis.

[0007]

A nucleic acid acting protein shares one or more cluster in common comprising basic amino acids, i.e., lysine and arginine. However, the composition of the basic amino acid in these clusters is irregular, and significantly differs among proteins. For example, histon protein contains quite a lot of lysine and arginine residues, while protamin contains little lysine residue (H. Busch, in: Histones and Other Nuclear Proteins, p.28, Academic Press., New York (1965)). These facts suggest that lysine and arginine may interact with the nucleic acid in a different mechanism. As a basic functional group, lysine has a primary amino group while arginine has a guanidino group.

[0008]

In physiological pH, the primary amino group and the guanidino

group are positively charged. It is reported that in physiological pH, the interaction of the peptide having high content of lysine or arginine with DNA is mainly ionic action, while the interaction of peptide having high content of arginine with DNA or RNA involves hydrogen bond that is stronger than ionic action (F. A. Cotton, V. W. Day, E. E. Hazen Jr. and S. Larsen, J. Am. Chem. Soc. 95, 4834 (1973); G. Lancelot, R. Mayer and C. Helene, Biochem. Biophys. Acta 564, 181 (1979); K. M. Weeks, C. Ampe, S. C. Schultz, T. A. Steitz and D. M. Crothers, Science 249, 1281 (1990)).

[0009]

Patent document 1: Japanese published unexamined application 2001-78769

Non patent document 1: A. Ferdous, T. Akaike and A. Maruyama, Bioconjugate Chem. 11, 520 (2000)

Non patent document 2: W. J. Kim, T. Ishihara, T. Akaike and A. Maruyama, Chem. Eur. J. 7, 176 (2001).

#### Summary of the invention

Problems to be solved by the invention

[0010]

As mentioned above, the inventor already has provided a cationic polymer as a substance that can accelerate nucleotide chain exchange reaction between a double stranded chain and a single stranded chain complementary thereto. However, in

order to further improve an analysis rate, and facilitate application to various fields of nucleic acid biotechnology and nanotechnology, development of a substance having further improved acceleration activity is still in need.

5 [0011]

In order to solve the problem, the inventors analyzed basically the function of conventional copolymers and succeeded in development of a substance having a higher accelerating activity, and accomplished the invention.

10

Means for solving the problems

[0012]

The present invention relates to a preparation for accelerating an exchange reaction between a nucleotide  
15 sequence at a specific site of a double stranded DNA or RNA and its homologous nucleotide sequence, the preparation comprising a cationic polymer having a guanidine group-containing main chain and a hydrophilic functional group, as an active ingredient.

20

Advantage of the invention

[0013]

The present invention has provided the having an exchange reaction accelerating activity of several ten to several  
25 hundred times as compared with that of conventional copolymers.

Thus, compared with the prior art, the nucleotide chain can be exchanged at a lower temperature and/or at a higher rate.

[0014]

The double stranded DNA or RNA refers to a chain that can maintain a higher-order structure formed between DNA or RNA with the paring of complementary base pairs (or nucleotide) of adenine (A), thymine (T), guanine (G) and cytosine (C) in case of DNA, and A, uracil (U) and G, C in case of RNA. In this invention, DNA or RNA as above may be isolated from biogenic substance, or present in a tissue section of a living body without isolation. Furthermore, a synthesized double-stranded DNA or RNA is also included.

[0015]

The nucleotide sequence at the specific site of DNA or RNA as above may be a nucleotide sequence of any sites, such as a sequence at an end or intermediate of a straight chain DNA or RNA. Its homologous nucleotide sequence means that the bases constituting their sequences are substantially identical. The phrase "substantially identical" means that a nucleotide sequence at the specific site mentioned above and its homologous sequence have at least 90%, preferably 95% identical segment of A, T and G, C, while T being optionally replaced by U.

[0016]

Therefore, an exchange reaction means that the nucleotide

sequence at the specific site of a single chain of the double stranded DNA or RNA comprising complementary two chains is replaced by its homologous nucleotide. Particularly, the exchange is carried out as follows: the nucleotide sequence at the specific site of the double stranded DNA is replaced by its homologous nucleotide sequence of the single stranded DNA or RNA; or the nucleotide sequence at the specific site of the double stranded RNA is replaced by its homologous nucleotide sequence of the single stranded DNA or RNA.

10 [0017]

The homologous nucleotide sequence in the single stranded DNA or RNA may comprise either the whole sequence of the single stranded DNA or RNA, or any part thereof.

[0018]

15 Exchange or an exchange reaction means that the nucleotide sequence at the specific site of the double stranded DNA or RNA is simply replaced and re-organized by its homologous nucleotide sequence of the single stranded DNA or RNA. The reaction does not therefore involve removal of the native  
20 nucleotide sequence at the specific site.

Brief description of the drawings

[0019]

Fig. 1 is a drawing that shows change over time of the  $^1\text{H}$  NMR spectrum during the reaction of guanidination of PLL-g-Dex.  
25



Fig. 2 is a drawing that shows  $^{13}\text{C}$  NMR spectrum of the copolymer (GPLL-g-Dex) obtained after purification.

Fig. 3 is a drawing that shows the measurement result of GPLL-g-Dex and PLL-g-Dex by Sec-MALS.

5 Fig. 4 is a drawing that shows change over time after guanidination of PLL-g-Dex copolymer and PLL homopolymer ( $M_n = 27,600$ ).

Fig. 5 is a drawing that shows UV melting curve and  $T_m$  value of 20 bp dsDNA in the presence or absence of comb-type copolymer  
10 at various N/P ratios.

Fig. 6 is a drawing that shows the result of  $T_m$  measurement of the affinity of the copolymer to DNA.

Fig. 7 is a photograph of electrophoresis gel image that shows the progress of chain exchange reaction.

15 Fig. 8 is a graph that shows the progress of a chain exchange reaction.

Fig. 9 is a photograph of electrophoresis gel that shows polyion complex forming ability of PLL-g-Dex and GPLL-g-Dex with ODN.

20 Best mode for carrying out the invention

[0020]

The guanidino group in the main chain that constitutes the cationic polymer according to the invention can have any structure known by those skilled in the art. Particularly,  
25 a guanidino group derived from arginine, one of amino acids,

is preferred. More preferably, the main chain has also a moiety derived from lysine.

Therefore, a particularly preferred example includes a cationic polymer having the main chain comprising a moiety  
5 obtained by guanidination of a polymer having a primary amino group or a secondary amino group.

The ratio of the residues having granidino group in the main chain of the cationic polymer according to the intended use may be appropriately selected by those skilled in the art, but  
10 particularly a ratio of 0.3 to 1 is preferred. That is, the ratio of arginine and lysine residues in the main chain of the cationic polymer is preferably 1:0 to 0.1:0.9, more preferably 1:0 to 0.3:0.7. The numbers of arginine and lysine residues contained in polyarginine and polylysine blocks may be  
15 appropriately selected by those skilled in the art, but are usually in the range of 10 - 5,000.

[0021]

Preferably, the cationic polymer according to the invention has a hydrophilic functional group in the side chain. The  
20 hydrophilic functional group may be any group well-known by those skilled in the art. Examples include water-soluble polyalkylene glycols such as polyethylene glycol; water-soluble polysaccharides such as dextran, the maltohexaose, pullulan, amylose, and arabinogalactan;  
25 water-soluble polyamino acid containing hydrophilic amino

acids such as serine, asparagin, glutamine, and threonine; water-soluble polymer synthesized using acrylamide and derivatives thereof as a monomer; water-soluble polymer synthesized using methacrylic acid, acrylic acid, or derivatives thereof (e.g., hydroxyethyl methacrylate) as a monomer; and polymer synthesized using one or more water-soluble polymer selected from a group consisting of polyvinyl alcohol and derivatives thereof.

[0022]

10 The side chain comprising the hydrophilic functional group such as hydrophilic polymer binds to an appropriate site such as the primary amino group or secondary amino group of the cationic polymer in a graft-shape according to a method well known by those skilled in the art.

15 [0023]

Further, the molecular weight of the cationic polymer, the chain length of the side modifying chain group itself, and the extent of grafting is not limited because the optimum values may vary depending on the intended use. However, those skilled in the art can select each optimum value with reference to the examples that will be described below.

For example, the molecular weight as a free salt may be 2,000 to 200,000, the content of graft-shape side chain derived from the hydrophilic polymer may be 30 to 90% by weight, and the grafting ratio may be 5 - 40%.

[0024]

The invention provides a preparation having the cationic polymer mentioned above as an active ingredient. The preparation can be constituted by crude or synthesized polymer or its purified polymer, and buffer or normal saline as necessary, provided that it will not have any adverse effects on the intended exchange reaction. The polymer may be a mixture having one or more types of the polymer.

[0025]

10 The cationic polymer as described will be generally selected to attain a charge ratio of 0.1 or more, preferably 0.5 to 1000 between the cationic moiety and the phosphate in total DNA or RNA (single stranded and double stranded) provided for the exchange reaction. On the other hand, the mole ratio of the  
15 single stranded DNA or RNA to be used in the exchange reaction for the double stranded DNA or RNA may be advantageously selected to be 5 or more.

[0026]

The exchange reaction can be carried out at a temperature in  
20 which the double stranded DNA or RNA will not be denatured by heat, and without need of a cofactor such as ATP or  $Mg^{2+}$ . Preferably the temperature may be about 5°C to 40°C, which is a physiologically acceptable temperature wherein no adverse effect on the cell of the organism occurs.

25 [0027]

The cationic polymer of the invention can efficiently exchange the nucleotide sequence at the specific site of the double stranded DNA or RNA for its homologous nucleotide sequence of the single stranded DNA and RNA under above condition.

5 Theoretically, the oligonucleotide has any chain length provided that it is 5 mer or longer. Preferably, the invention may be applied to a sequence having a length between 10 and 500 mer. Therefore, the preparation of the cationic polymer of the invention is useful in hybridization of a primer or probe,  
10 or an oligonucleotide carrying a known label or agent with the double stranded DNA or RNA; and in PCR or RT-PCR process or a process using a DNA chip that utilizes the feature of a primer, probe or oligonucleotide hybridized to a specific site of the double stranded DNA or RNA, and in nucleic acid analysis that  
15 utilizes fluorescence in situ hybridization.

[0028]

In addition, the cationic polymer according to the invention is also useful in accelerating the exchange between a specific site of the double stranded mRNA and an antisense  
20 oligonucleotide, ribozyme, deoxyribozyme, or oligonucleotide for forming a triple strand that contains a sequence complementary thereto so as to improve gene control function of these oligonucleotides. The cationic polymer according to the invention is also useful in controlling or regulating the  
25 replication, transcription or expression process by

exchanging a specific site of the genomic double stranded DNA for a nucleotide chain complementary thereto (by controlling the binding of a DNA binding protein).

## 5 Examples

[0029]

Next, the invention will be described in detail with reference to examples, however, it should be appreciated that the invention will not be limited in any way by these specific  
10 examples.

[0030]

Poly (L-lysine) (PLL, Mn = 27,600) was purchased from Nacalai Tesque Inc (Kyoto, Japan). Dextran T-10 (Dex, Mn = 8,700) was purchased from the Pharmacia BioTech Inc. (Uppsala, Sweden).

15 1-guanyl-3,5-dimethyl pyrazole nitrate ester (GDMP) as guanidinating agent was purchased from Sigma Aldrich Inc. (St. Louis, United States).

Oligonucleotide (ODN) of HPLC purification grade was purchased from Fasmac Co., Ltd. (Kanagawa, Japan), and its purity was  
20 checked by reverse phase HPLC.

[0031]

Preparation of poly(L-lydine)-graft-dextran (PLL-g-Dex)  
comb-type copolymer

PLL-g-dexedrine comb-type copolymer was prepared according to  
25 a method described in A. Maruyama, M. Katoh, T. Ishihara and

T. Akaike, Bioconjugate Chem, 8, 3 (1997) and A. Maruyama, H. Watanabe, A. Ferdous, M. Katoh, T. Ishihara and T. Akaike, Bioconjugate Chem. 9,292 (1998).

[0032]

- 5 Particularly, the copolymer was prepared by the reductive amination reaction of PLL-HBr by Dex in borate buffer, and purified by ultrafiltration using Saltrius ultrafiltration machine (MWCO 20000, Saltrius AG, Goettingen, Germany). The resulting copolymer was freeze-dried and its molecular weight  
10 was finally determined using SEC-MALS and  $^1\text{H}$ -NMR (as free salt,  $M_n=95,000$ , Dex content=82.9% by weight, grafting ratio Dex=7.4%).

[0033]

#### Guanidination of PLL-g-Dex comb-type copolymer

- 15 The guanidination of PLL-g-Dex is shown in the following scheme  
1. PLL-g-Dex (10mg, 13.3  $\mu\text{mol}$  (primary amino group), and GDMP (13.4mg, 66.6  $\mu\text{mol}$ ) were dissolved independently in 400  $\mu\text{L}$ . Each solution was adjusted to pH 9.5 by 5M NaOH in  $\text{D}_2\text{O}$  (final concentration of the primary amino group: 13 mM, final  
20 concentration of GDMP: 67 mM). Solutions were mixed, and put into a NMR sample tube. In order to evaluate change over time of guanidination of PLL-g-Dex, the reaction was carried out at 37°C in a NMR spectrometer (96 hours), while the change over time of  $^1\text{H}$ -NMR spectrum was recorded (Fig. 1).

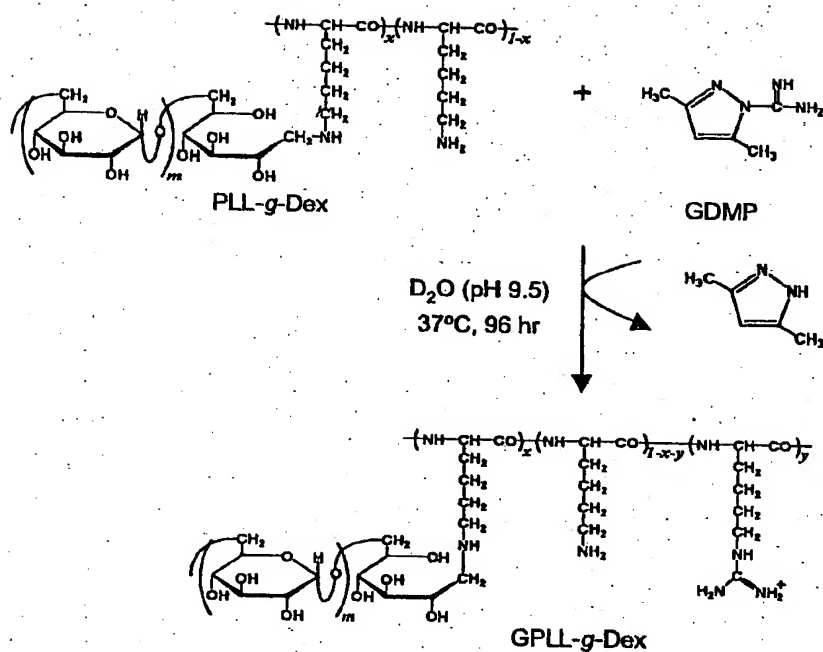
25 [0034]

It was expected that the peak (3.2 - 2.7 ppm range) of  $\epsilon$ -methylene proton would shift by guanidination. As shown in Fig. 1, with progress of the reaction, the peak of  $\epsilon$ -methylene proton at 2.8 ppm declined, and the peak of  $\epsilon$ -methylene proton at 3.2 ppm corresponding to the peak of  $\epsilon$ -methylene proton at the guanidinated site of lysine gradually increased. Finally, the peak of  $\epsilon$ -methylene proton disappeared. This indicates that the primary amino group is totally substituted with guanidino group during reaction for 96 hours. The resultant copolymer was separated by dialysis to Milli-Q water using Spectra/Por7 film of MWCO 25,000 (Spectrum Lab., Rancho Dominguez, USA), to remove unreacted GDMP. Guanidinated PLL-g-Dex (GPLL-g-Dex) was obtained by subsequent freeze-dry (yield 84.3%). Fig. 2 shows  $^{13}\text{C}$ -NMR spectrum of the copolymer obtained after purification. A new peak (g') appears at 156.6 ppm, which clearly indicates that the guanidino group was incorporated in the copolymer. Furthermore, as no unexpected change was observed in  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectrum, side reaction during guanidination can be neglected.



[0035]

Formula 1



15

[0036]

The resultant copolymer was dissolved in D<sub>2</sub>O (99.95% D; Merk Darmstadt, Germany).

20 <sup>1</sup>H-NMR spectrum (270 MHz) was determined using JEOL JNM-EX270 spectrometer (JEOL, Ltd., Tokyo, Japan) at the probe temperature of 25°C or 37°C. Chemical shift is expressed as ppm using 3-trimethyl silyl propionated sodium-2,2,3,3-d<sub>4</sub> (TSP) as an external control. The guanidination was

25 calculated from the peak area of ε-methylene protone of PLL.

After purification of GPLL-g-Dex,  $^{13}\text{C}$ -NMR spectrum (125 MHz) was determined using JEOL JNM-GX500 spectrometer (JEOL Ltd., Tokyo, Japan) by at 25°C, using TSP as an external control. [0037]

5 Size exclusion chromatography multiple angle light scattering (Sec-MALS)

The resultant copolymer was analyzed using size exclusion chromatography (SEC) system (Jasco model 800 Tokyo, Japan), on the Shodex OHpak SB-804 and 806 M column connected to a multiple angle light scattering (MALS) detector (Dawn-EOS, Wyatt Technology, Santa Barbara, CA) and reflective index (RI) detector. As a mobile phase, 0.5 M acetic acid aqueous solution and 0.2M  $\text{Na}_2\text{SO}_4$  aqueous solution were used at 25°C, 0.8 mL/min. Aliquot (100  $\mu\text{L}$ ) of graft copolymer solution (10 mg/mL) was injected into the column. Number average molecular weight ( $M_n$ ) and weight average molecular weight ( $M_w$ ) were calculated from RI and MALS signals using Astra (Wyatt Technology) for Windows (trade mark) Ver. 4.90. Values of  $dn/dc$  were determined by assuming that the collection of specimen from the column was 100%.

[0038]

The measurement result by Sec-MALS of GPLL-g-Dex and PLL-g-Dex is shown in Fig. 3. The molecular weight and its distribution determined by MALS (Fig. 3b) showed little change. Since PLL content in copolymer is only 17.1 % by weight, theoretically,

the molecular weight of copolymer increases by 5% by  
guanidination of PLL-g-Dex. Therefore, it is rational that  
guanidination of copolymer was hardly detected by the change  
in molecular weight. On the other hand, Fig. 3b clearly shows  
5 that significant fragmentation of PLL or Dex chain did not occur  
during guanidination.

[0039]

However, elution of GPLL-g-Dex was slightly delayed by  
guanidination (Fig. 3a). The delay might be caused by change  
10 in conformation of copolymer between copolymer and fillers,  
or change in interaction between copolymer and column packing.

[0040]

The change over time in guanidination of PLL-g-Dex copolymer  
and PLL homopolymer ( $M_n = 27,600$ ) at 37°C is shown in Fig. 4.

15 The conversion rate of PLL and PLL-g-Dex reached to 96% or more  
during the reaction for 45 hours. However, the reaction rate  
of PLL was higher than that of PLL-g-Dex. Since GDMP is a  
low-molecular-weight reagent, this difference cannot be  
attributed to the effect of steric constraint of grafted Dex  
20 chain. Rather, it may be caused by change in microenvironment  
such as dielectric constant around PLL backbone by grafted Dex  
chain.

[0041]

#### Measurement of UV melting curve

25 The nucleotide sequence of ODN used for the following UV melting

curve measurement was as follows: ODN 1: 5'-TCC TCG CCC TTG CTC ACC AT-3'; ODN 2: 5'-ATG GTG AGC AAG GGC GAG GA-3'. The double stranded DNA was prepared by mixing an equimolar amount of ODN1 and ODN2, annealed at 95°C for 5 minutes, and gradually cooled to a room temperature over 16 hours. Other solvents and chemicals of a reagent grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and used without further purification.

[0042]

10 Preservation solution was prepared by dissolving nucleotide in buffer I (10 mM PBS (pH 7.2) containing 150 mM NaCl and 0.5 mM EDTA). The concentration of the undiluted solution was calculated using the molar absorbance coefficient at 260 nm ( $1.65 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$  (ODN1),  $2.11 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$  (ODN2)). The solution for UV melting curve measurement was prepared by diluting the preservation nucleotide solution (final concentration 27.8  $\mu\text{M}$ ) with buffer I. The mixture of the diluted nucleotide solution was heated at 90°C for 5 minutes, then gradually cooled and was left at a room temperature for 16 hours. The undiluted solution of the copolymer was diluted with buffer I to obtain various concentrations. The diluted solution of nucleotide and copolymer was mixed by micropipet to obtain various N/P ratios ([amino group] copolymer/[phosphate group] DNA).

25 [0043]

UV spectrum and UV melting curve were recorded by Shimadzu UV-1600 PC spectrometer equipped with TMSPC-8 thermoregulator (Shimadzu Corporation, Kyoto, Japan).

UV melting curve was recorded by 1K/min as heating rate.

- 5 Differential absorbance ( $\Delta A = A_{260} - A_{340}$ ) was calculated to correct baseline shift. The first derivative  $[d(\Delta A)/dT]$  was calculated from melting curve data. The peak temperature in the derivative curve was defined as the melting temperature ( $T_m$ ).

10 [0044]

Influence of guanidination on interaction between DNA/copolymer

- Influence of guanidination on interaction between DNA/copolymer was analyzed by dissolution analysis of double stranded DNA. Fig. 5 indicates UV melting curves and  $T_m$  values of 20 bp dsDNA under the presence or absence of the comb-type copolymer at various N/P ratio from 0 (DNA only) to 10. When DNA only, transition from double strand to single strand was observed at 70.3°C in physiological ionic strength (10 mM PBS - 150 mM NaCl). Increase in  $T_m$  of dsDNA was observed under a condition where either PLL-g-Dex or GPLL-g-Dex was present. The increase of  $T_m$  reached to plateau at N/P ratio 2, and remained same until N/P ratio 10 (Fig. 5b). PLL-g-Dex increased  $T_m$  by 15°C, while GPLL-g-Dex increased  $T_m$  by 5°C only.
- 25 It is considered that the comb-type copolymer stabilizes

double stranded and triple stranded DNA mainly by suppressing the static electricity repulsive force in DNA chain (A. Maruyama, M. Katoh, T. Ishihara and T. Akaike, Bioconjugate Chem, 8, 3 (1997); A. Maruyama, H. Watanabe, A. Ferdous, M. Katoh, T. Ishihara and T. Akaike, Bioconjugate Chem. 9, 292 (1998); A. Maruyama, Y-I. Ohnishi, H. Watanabe, H. Torigoe, A. Ferdous and T. Akaike, Colloids Surf. B 16, 273 (1999)). Therefore, from the result in Fig. 5, the affinity of the ion of GPLL-g-Dex to dsDNA may be weaker than that of PLL-g-Dex.

10 [0045]

Next, the affinity of the copolymer to the DNA was evaluated by  $T_m$  measurement (Fig. 6). The solutions of PLL-g-Dex and GPLL-g-Dex were put into a DNA solution together to obtain predetermined N/P ratio. The N/P ratio of PLL-g-Dex was adjusted to 2, 4, and 8, while the N/P ratio of GPLL-g-Dex was kept at 2. It should be noted that  $T_m$  showed little increase under the presence of GPLL-g-Dex, although an excessive concentration of PLL-g-Dex was present (up to 4 times GPLL-g-Dex as shown in Fig. 6b). These results show the selective interaction of dsDNA and GPLL-g-Dex, and mean that the affinity of GPLL-g-Dex to dsDNA is much higher than that of PLL-g-Dex.

[0046]

The above result suggests the possibility that the stability of dsDNA is influenced not only by affinity to dsDNA but also

25

by interaction in other mode. It was reported that affinity of oligoarginine to DNA is stronger than that of oligolysine. Both oligoarginine and oligolysine mainly interact with DNA via ionic interaction. However, hydrogen bond interaction contributes to the strong interaction between oligoarginine and DNA (D. P. Mascotti and T. M. Lohman, Biochemistry 36 and 7272 (1997)). Similarly, the stronger affinity of GPLL-g-Dex than PLL-g-Dex may be attributable to the hydrogen bond interaction between guanidino group and DNA. The involvement of the hydrogen bond between copolymer and DNA may cause weaker stabilizing effect of GPLL-g-Dex to dsDNA. These findings are in line with a finding disclosed in a conventional study that arginine in dipeptide Arg-Glu can form hydrogen bond with nucleic acid phosphate like cytosine in single stranded DNA, and guanosine in single stranded or double stranded DNA (G. Lancelot, R. Mayer and C. Helene, Biochem, Biophys. Acta 564,181 (1979)).

[0047]

#### Analysis of chain exchange reaction

The base sequences of ODN used for the following analysis experiment of a chain exchange reaction were as follows: ODN1 (5'-TCC TCG CCC TTG CTC ACC AT-3'); ODN2 (5'-ATG GTG AGC AAG GGC GAG GA- 3'); ODN3 (5'-ATG GTG AGC AAG GGC GAG GA-3' (FITC)). In the solution of the double stranded DNA, an equimolar amount of ODN1 and ODN3 were mixed, annealed at 95°C for 5 minutes,

and slowly cooled to a room temperature over 16 or more hours for preparation. In the solution of the single stranded DNA, ODN2 was used as a sample. Other solvents and chemicals of a reagent grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and used without further purification.

[0048]

Preservation ODN solution was prepared by dissolving ODN in buffer I (10 mM PBS (pH 7.2) containing 150 mM NaCl). The concentration was calculated based on the molar absorbance coefficient at 260 nm. ( $1.65 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$  (ODN1) and  $2.11 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$  (ODN2, ODN3)). The undiluted solutions of PLL-g-Dex and GPLL-g-Dex as copolymer were dissolved with buffer I for preparation. The concentration was calculated based on the average molecular weight.

[0049]

The solution for analysis of chain exchange reaction rate was adjusted to the intended concentration by diluting these preservation solutions with buffer I.

First, the solution of the double stranded DNA and the solution of copolymer were mixed to be  $N/P = 2$ . Next, the solution of the single stranded DNA was added to start chain exchange reaction (final concentrations were, 0.5  $\mu\text{M}$  for double stranded DNA, 2.5  $\mu\text{M}$  for single stranded DNA for exchange).

The reaction temperature was 15°C.



When the intended reaction time (up to 6 hours) was reached, in order to negate the interaction between ODN and copolymer to stop the chain exchange reaction, an excessive amount of Salmon sperm DNA was added. In gel electrophoresis, 13% polyacryloamide gel as electrophoresis gel, and TBE buffer solution (reagent containing EDTA in Tris borate buffer) as electrophoresis buffer were used. After loading an ODN sample to this gel for electrophoresis, the electrophoresis was carried out under condition of a room temperature for one hour at 100V.

[0050]

After electrophoresis, electrophoresis gel was obtained that shows the double stranded ODN3 and the single stranded ODN3 by fluorescence band by FITC. The transition from the double strand to the single strand indicates the progress of chain exchange reaction.

Using LAS-3000 fluorescence image analyzer (Fuji Photo Film Co., Ltd., Kanagawa, Japan), the electrophoresis gel (Fig. 7) was imaged to quantify the abundance ratio of the fluorescence band. The abundance ratio of this fluorescence band was used as the chain exchange rate (Fig. 8).

As a result, the difference in influence on chain exchange reaction was observed between PLL-g-Dex and GPLL-g-Dex. The result clearly showed that the GPLL-g-Dex raised the exchange rate more than PLL-g-Dex.

[0051]

Analysis of polyion complex formation

The ODN base sequences used for the analysis experiment of polyion complex formation (IPECs: InterPolyElectrolyte Complexes) were as follows: ODN1 (5' -TCC TCG CCC TTG CTC ACC AT-3'); ODN3 (5'-ATG GTG AGC AAG GGC GAG GA-3' (FITC)). In the double stranded DNA solution, an equimolar amount of ODN1 and ODN3 were mixed, annealed at 95°C for 5 minutes, and slowly cooled to a room temperature over 16 or more hours for preparation. In the single stranded DNA solution, ODN3 was used as a sample. Other solvents and chemicals of a reagent grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and used without further purification. Preservation ODN solution was prepared by dissolving ODN in buffer I (10 mM PBS (pH 7.2) containing 150 mM NaCl). The concentration was calculated based on the molar absorbance coefficient at 260 nm. ( $1.65 \times 10^5 \text{M}^{-1}\text{cm}^{-1}$  (ODN1),  $2.11 \times 10^5 \text{M}^{-1}\text{cm}^{-1}$  (ODN3)). The undiluted solution of PLL-g-Dex and GPLL-g-Dex as copolymer were dissolved by buffer I for preparation. The concentration was calculated based on the average molecular weight.

[0052]

The solution for analysis of IPECs formation was adjusted to the intended concentration by diluting these preservation solutions with buffer I.

A mixed solution of the double stranded DNA (final concentration: 0.5  $\mu$ M) and the single stranded DNA (final concentration: 1.0  $\mu$ M) was added to the solution of copolymer at intended N/P ratio (0.0 - 2.0). Then the mixture was reacted

5 at a room temperature for one hour to form IPECs.

In gel electrophoresis, 13% polyacrylamide gel as electrophoresis gel, and TBE buffer solution (reagent containing EDTA in Tris borate buffer) as electrophoresis buffer were used. After loading an ODN sample to this

10 electrophoresis gel, electrophoresis was carried out under condition of a room temperature for one hour at 100V.

[0053]

After the completion of electrophoresis, electrophoresis gel was obtained that showed existence of free ODN3 by fluorescence

15 band by FITC. Disappearance of fluorescence band in both the double strand and the single strand status indicates the extent of IPECs formation.

Using LAS-3000 fluorescence image analyzer (Fuji Photo Film Co., Ltd., Kanagawa, Japan), the electrophoresis gel (Fig. 9)

20 was imaged.

As a result, the difference in the polyion complex formation ability of PLL-g-Dex to ODN, and that of GPLL-g-Dex to ODN was observed. Both PLL-g-Dex and GPLL-g-Dex formed complete complex to the double stranded DNA at N/P ratio of 1 or more,

25 without significant difference. However, to the single

stranded DNA, the binding ability of GPL-rg-Dex was higher than that of PLL-g-Dex.

#### Industrial applicability

5 [0054]

The preparation according to the invention can be used in many fields, including gene analysis (SNPs analysis, DNA chip, PCR, etc.), a nano structure using nucleic acid, molecule machine, and nucleic acid medicine.